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14. ABSTRACT
Each person's cancer is as unique as his or her fingerprint, which explains unpredictable responses to therapies and poses new biotechnology challenges for tumor characterization on the molecular level. For these reasons, it is of pivotal importance to develop novel molecular profiling methodologies for diagnosis, prognosis and individually tailored therapeutics of patients based on the biology of their tumors. We proposed to develop oligonucleotide tagged quantum dots and antibodies for multiplexed imaging of prostate cancer samples.

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Final Report: PC061345-Molecular Profiling of Prostate Cancer Specimens Using Multicolor Quantum Dots

PI: X. Gao, Department of Bioengineering, University of Washington

Introduction

There is increasingly compelling evidence that cancer varies both genetically and phenotypically between patients who have identical histologic and tissue types and stages of cancer. Each person's cancer appears to be as unique as his or her fingerprints. This uniqueness helps explain the variable and unpredictable responses of tumors in individual patients to therapies. The prognosis and choice of therapy for prostate cancer is currently based mainly on three parameters obtained at the time of diagnosis - clinical stage, serum prostate specific antigen, and the Gleason grade of the cancer. The grade which is based on microscopic tumor architecture, has a value between 2 (well differentiated and indolent) and 10 (poorly differentiated and rapidly progressive).[1] Studies have demonstrated a direct correlation between grade and clinical measurements of disease outcome, including time to tumor recurrence and probability of dying of tumor. However, the Gleason system has limitations. It is (1) subject to interobserver variability; (2) does not stratify patients into a large number of categories (>85% of tumors are grade 6 or 7); and (3) does not provide molecular information. Molecular biomarkers that can be localized in needle biopsy tissue using immunostains have been developed to better predict the biology of prostate cancers. Knowing the molecular profile of a prostate cancer raises the prospect of therapy targeted to specific molecules. We proposed a multivariate molecular imaging approach for prostate cancer molecular profiling using oligonucleotide tagged semiconductor quantum dots and specific antibodies as shown in **Figure 1**.

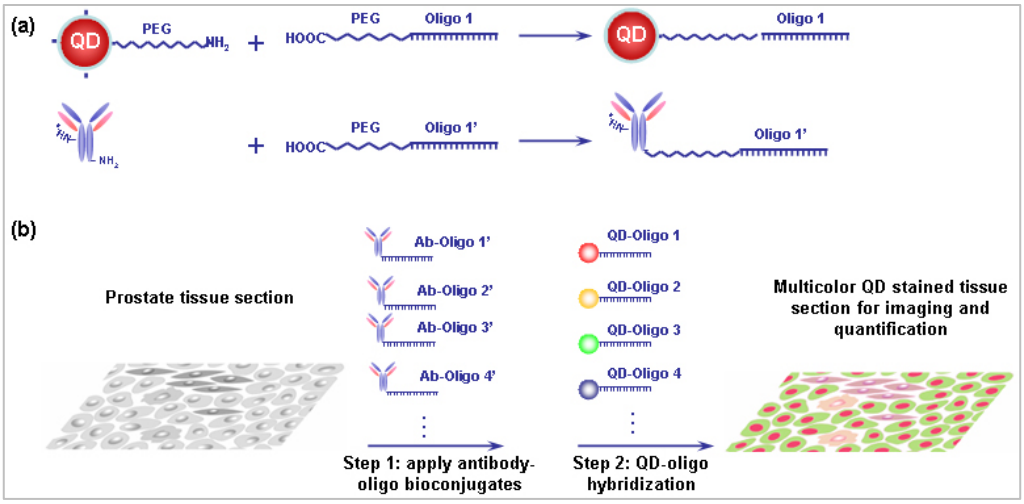
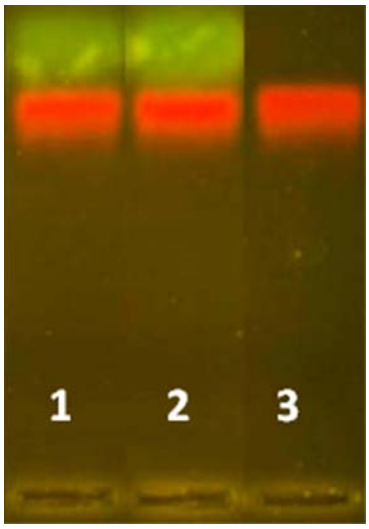


Figure 1. Schematic illustration of FINISH technology. (a) to prepare FINISH probes, QDs and antibodies are first tagged with complimentary pairs of oligonucleotides via covalent bond. (b) a two-step assay of first applying all the antibody probes, then multicolor QDs tagged with comlimentary oligonucleotides. If the target is present, hybridization will occur and vice versa. Thus, the antibody binding assay is transformed into multiplexed and robust DNA hybridization.

Body

In the past two year, we successfully developed the FIHISH technology. We started with QD preparation, QDs with emission wavelength ranging from 480-650 nm with high quantum yield have been synthesized based on literature procedures.[2-5] The QDs were characterized by optical absorption, fluorescence emission, transmission electron microscopy, and dynamic light scattering, to ensure sufficient quantum yield and monodispersity. The resulting QDs were not water-soluble. We have identified two novel polymers that are not only able to convert them into hydrophilic nanoparticles but also reduce non-specific binding due to the zwitterions feature of the polymer.

The first polymer we identified is PMAT. TOPO-coated hydrophobic QDs were dissolved in chloroform and mixed with 250-500 molar excess of polymer (PMAT). Few drops of methanol were added to chloroform to aid in dissolution of the polymer. After thorough mixing, chloroform was evaporated under vacuum to deposit PMAT molecules on the QD surface and yield polymer-coated QDs. Resulted powder was resuspended in chloroform, and 250-500 molar excess of diamine cross-linker was added. Spontaneous reaction between primary amines of cross-linkers and anhydride rings of the polymer covalently bound neighbor PMAT molecules on the QD surface, thus producing stable coating. Polymer encapsulation of QDs without further cross-linking also produced water-soluble particles; however, chemical modification of such particles often resulted in severe aggregation due to exposure of hydrophobic patches or unbound PMAT on QD surface. After 30 minutes incubation with cross-linker, chloroform was evaporated under vacuum. Resulted powder was resuspended in 50 mM Borate buffer (pH 8.5). Basic buffer promoted opening of anhydride rings into carboxylic acid groups, which yielded particles water-soluble and highly negatively charged at neutral or basic pH. Polymer-coated QDs were purified by 2 rounds of ultra-centrifugation at 45000 rpm for 45-60 minutes. Soft pellet (100-150 uL) was collected each time and resuspended in Borate buffer. We also systematically investigated the purification schemes, and it was interesting to note that several purification methods reported in literature, such as Superdex-75 gel column and 100 kDa concentrator, failed to remove excess polymer from solution, as was indicated by presence of PMAT fluorescence peak and polymer band in agarose gel (Fig. 1). Even though these methods are expected to be effective in removing single polymer molecules with molecular weight of only 9 kDa, it is likely that PMAT forms high-MW micelles in aqueous buffers (with ~3 nm hydrodynamic radius), that are hard to separate from QDs using methods based on particle size.



1 – gel column
2 – concentrator
3 - ultracentrifuge

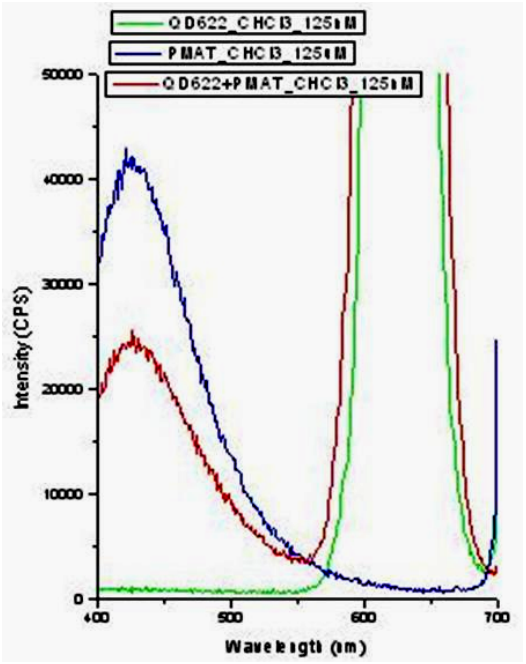


Fig. 1: PMAT presence in solution can be detected by gel electrophoresis with SYBR-Gold staining (left) and its fluorescence (right). Both purification with Supredex-75 gel column and 100K concentrator had significant amount of polymer in solution, and only ultracentrifugation yielded pure QDs.

PMAT-coated QDs had hydrodynamic diameter of 10-13 nm and quantum yield of 50-55% (only slight drop from 60% QY of TOPO-coated QDs in chloroform). When run on 1% agarose gel at 100V for 30 minutes, QDs formed sharp band moving towards positive electrode, indicating monodisperse population of negatively-charged particles. Potential for further particle functionalization was demonstrated by EDC-mediated covalent conjugation of streptavidin and Protein A to carboxylic acid groups on QD surface. Successful conjugation was confirmed by reduction in electrophoretic motility and increase in hydrodynamic size.

While highly stable, water-soluble, and easy to modify, PMAT-coated QDs showed non-specific binding to fixed cells, thus precluding from using such particles for preparation of probes for cell and tissue staining. Permeabilization of specimens with charged (DTAC and SDS) and neutral (Triton X-100 and Tween-20) detergents and thorough blocking with BSA did not significantly reduce non-specific staining. Alternative strategy of coating hydrophobic QDs with zwitterionic polymer PMAL-C8 was then studied.

QD encapsulation with PMAL-C8:

PMAL-C8 is also an amphyphilic polymer like PMAT, but it has several features different from PMAT. First, it has alternating carboxylic acid groups and tertiary amines, which provide

zwitterionic surface neutrally charged at slightly basic pH. Second, it has C8 hydrophobic side-chains matching the length of TOPO molecules, thus yielding compact deposition of polymer on QD surface and producing stable coating even without further cross-linking. Following procedure developed for PMAT coating, QDs were coated with PMAL-C8 by mixing nanoparticles and polymer in chloroform with 250-500 molar excess of polymer and evaporating solvent under vacuum. Resulted powder was dissolved in 50 mM Borate buffer (or water or PBS), filtered through 0.22 um syringe filter, and purified by 2 rounds of ultracentrifugation at 45000 rpm for 60 minutes. PMAL-coated QDs had hydrodynamic diameter from 7nm (for green QD513) to 12nm (for red QD622), preserved quantum yield, and no electrophoretic motility. Most notably, such QDs showed minimal non-specific interaction with fixed cells (Fig. 2), thus providing a suitable platform for further preparation of probes for cell and tissue staining.

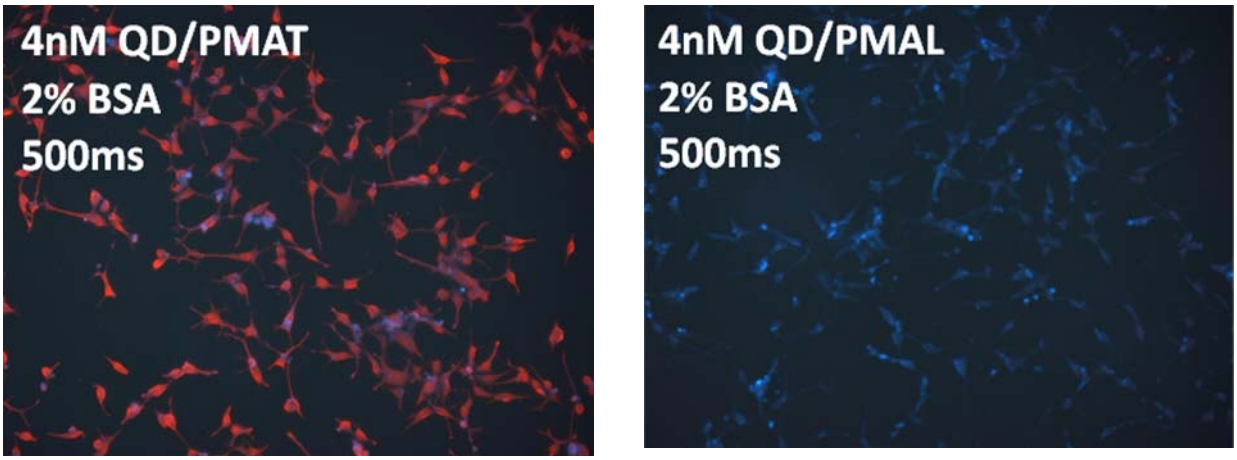


Fig. 2: PMAT-coated QDs (left) show very high non-specific binding to fixed cells, precluding from utilization of such particles in staining applications. PMAL-coated QD (right), on the other hand, show minimal non-specific interaction, providing suitable platform for preparation of probes for cell staining.

In parallel to the nanoparticle synthesis, we have also designed and synthesized multiple oligonucleotide sequence pairs (16 base long) that have approximately the same melting temperatures (T_m) and no or minimum similarity to endogenous DNA in mammalian cells using commercially available software and database (IDT oligo design & NCBI BLAST). In addition, to minimize non-specific binding and potential steric hindrance from nanoparticles, each oligonucleotide strand was inserted with a polyethyleneglycol (PEG) spacer.

Conjugation of amine-modified oligonucleotides to QD/PMAL

We then proceeded with the bioconjugation. Oligonucleotides were custom-synthesized to have 54-unit PEG spacer and primary amine on C6-linker at 5' end, thus providing anchor point (primary amine) and flexible hydrophilic arm (PEG54). Concentration of oligonucleotides was confirmed by measuring absorption peak at 260 nm. Presence of primary amines was confirmed by colorimetric TNBS test. It was noticed that color development was more pronounced when test was performed

in DMSO solution vs. aqueous Bicarbonate buffer, thus indicating that primary amines were not accessible in aqueous buffer. This behavior might be explained by the presence of hydrophobic C6 linker directly attached to NH2 group. Such hydrophobic region could be easily hidden by hydrophilic PEG spacer, restricting access to amine group. Therefore, it was proposed that chemical conjugation between QDs and oligos had to be done in DMSO or DMF solution.

Carboxylic acid groups on the PMAL molecules provided points of chemical functionality on QD surface. However, unlike PMAT-coated QDs, access to carboxylic acid groups on PMAL is sterically hindered by abundance of bulky tertiary amines spaced away from the QD surface by C3 linkers. Therefore, while providing very good barrier from non-specific interaction with biomolecules, chemical modification of such surface proved to be challenging. Yet, since NH2 group is placed on C6 linker on oligonucleotides, it should be able to reach carboxylic acid groups between tertiary amines.

EDC-mediated conjugation between PMAL-coated QDs and amine-modified oligonucleotides was performed in ~100% DMSO or DMF solution. Unlike PMAT-coated QDs that showed severe aggregation in DMSO/DMF, QD/PMAL remained single even after addition of small amount of EDC. Some aggregation could happen upon addition of larger amounts of EDC; however, upon resuspension in Borate buffer aggregates broke back into single QDs. QDs were mixed with 10-50 molar excess of oligonucleotides in DMSO/DMF and small amount of EDC was added. Reaction was incubated at room temperature for 4 hours, dissolved in Borate buffer 1:10 to yield ~10% DMSO/DMF solution, and unbound oligonucleotides and EDC were removed by 6 cycles of concentration/dilution (1:10 dilution each cycle) using 100K MWCO concentrator. Successful conjugation was confirmed by detection of absorption peak at 260 nm in QD-oligo solution and increase in hydrodynamic size from 10nm to 11-13 nm (depending on number of oligos conjugated) (Fig. 3A and 3C). Interestingly, conjugation reaction with identical conditions performed in Bicarbonate buffer did not produce any QD-oligo conjugates (Fig. 3B and 3D), thus confirming hypothesis that C6-NH2 groups are not accessible in aqueous environment. Based on the oligonucleotide and QD absorption it was calculated that 3-4 oligonucleotides were conjugated to each QD.

In order to test staining functionality of QD-oligo bioconjugates, fixed LnCap cells were non-specifically labeled by non-complimentary and complimentary oligonucleotides and QD-oligo conjugates were applied at QD concentration of 4nM in 2%BSA/Borate buffer. After 1 hour incubation at room temperature and thorough washing with Borate buffer only cells labeled with complimentary oligonucleotide showed significant staining (Fig. 4), indicating that QD-oligo conjugates were functional and specific.

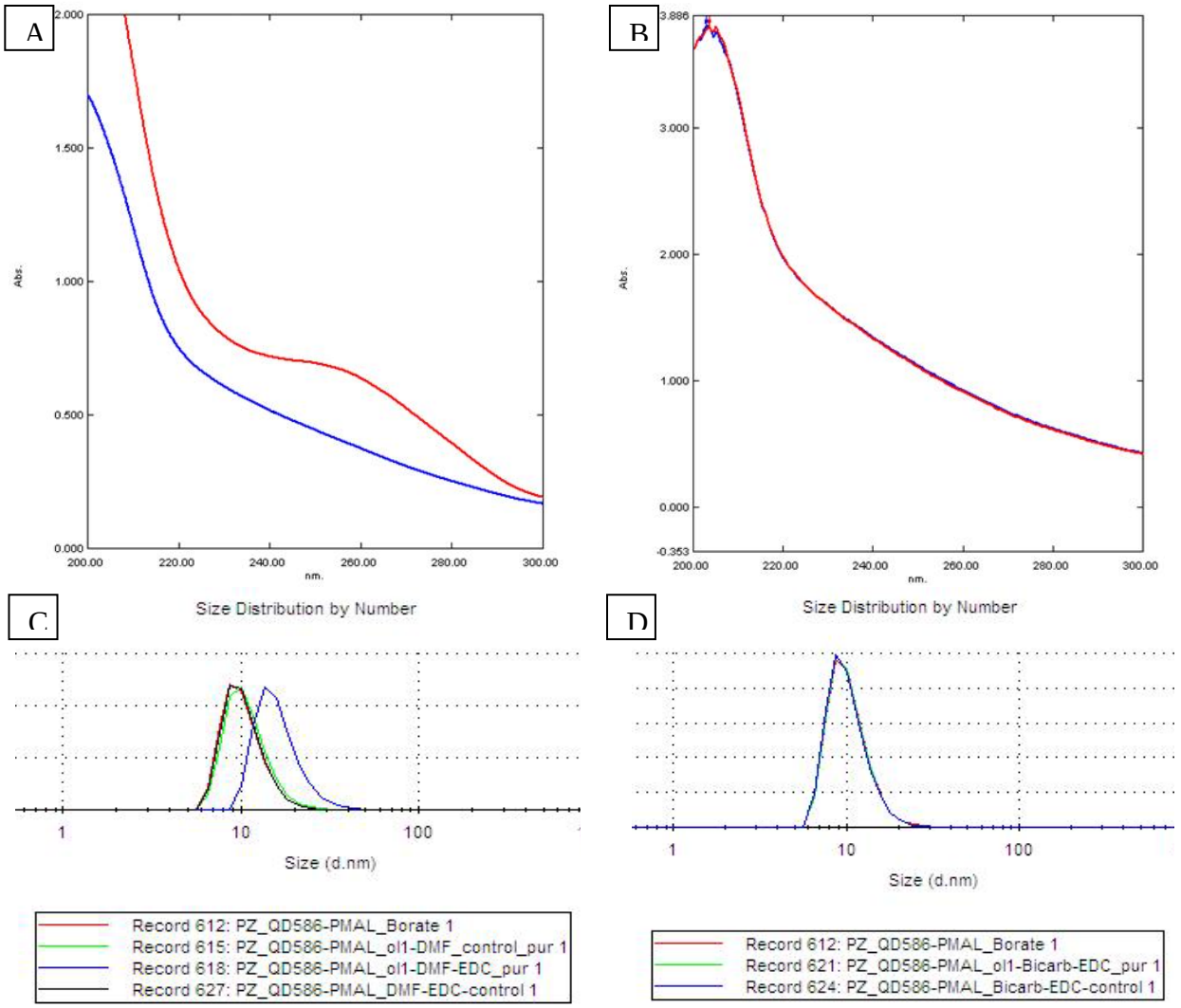


Fig. 3: Covalent conjugation of amine-modified oligonucleotides to PMAL-coated QDs. QD-oligo conjugates prepared in ~100% DMF showed distinct DNA absorption peak after purification (A) and increase in hydrodynamic size corresponding to deposition of oligonucleotide-PEG on the QD surface (C). Reaction performed under identical conditions in aqueous Bicarbonate buffer failed to produce any QD-oligo conjugates showing no changes in light absorption (B) and particle size (D) in purified samples vs. control.

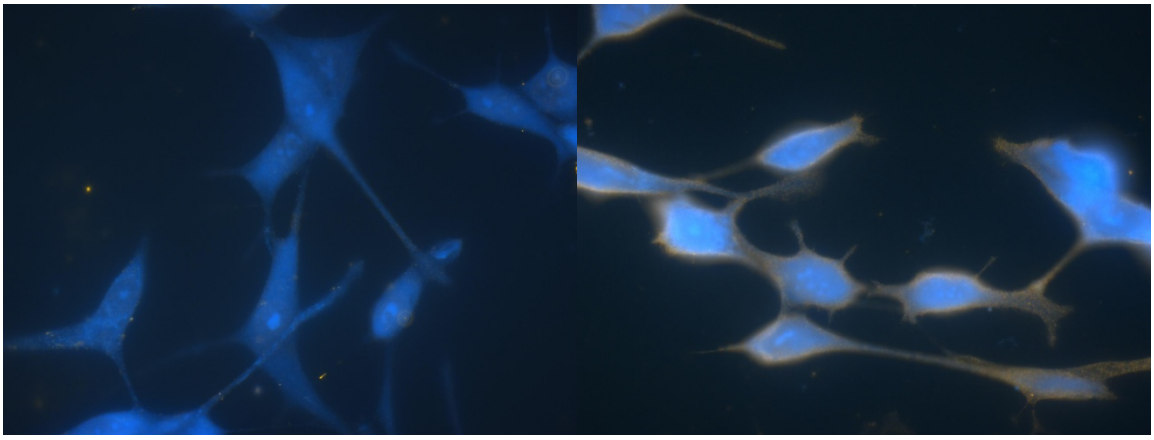


Fig. 4: Test of staining functionality of QD-oligo conjugates shows no non-specific staining of LnCap cells labeled with non-complimentary oligonucleotides (left) and strong specific membrane staining (orange) of cells labeled with complimentary DNA (right). Blue color indicates cell autofluorescence.

Conjugation of amine-modified oligonucleotides to primary antibodies

In order to specifically label certain biomarkers with oligonucleotides, amine-modified oligos were covalently conjugated to primary antibodies. Mouse anti-AR (androgen receptor), mouse anti-Her2, and rabbit anti-MAOA (monoaminooxidase A) antibodies were first purified from BSA by 5 runs in 100kDa MWCO concentrators and then activated by SANH cross-linker, which converted primary amines on antibodies into hydrazide residues reactive towards aldehydes. Reaction was performed in PBS with ~250 molar excess of SANH added (SANH was initially dissolved in DMSO and then ~1uL was added to solution to yield no more than 1% final DMSO content) and incubated for 4 hours at room temperature. Excess cross-linker was removed by either Zeba Protein Desalting Spin Column or 5 runs in 100kDa concentrator with exchange of buffer to Citrate pH6.

Amine-modified oligonucleotides were in turn reacted with SFB, which converted primary amines into aldehydes. Reaction was performed in PBS with ~500 molar excess of SFB added (SFB was dissolved in DMSO and ~1uL was added to solution) and incubated for 4 hours at room temperature. Excess SFB was removed by either Zeba Protein Desalting Spin Column or 3 runs in 3kDa concentrator with exchange of buffer to Citrate pH6.

Activated antibodies were mixed with modified oligonucleotides in Citrate buffer with ~10-20 molar excess of oligos and incubated at room temperature overnight. Unreacted oligonucleotides were removed by 8 runs in 100kDa concentrator (with 1:10 dilution each run) with buffer exchange to PBS. Successful conjugation was confirmed by detection of DNA absorption peak at 260 nm in purified IgG-oligonucleotide solution. To test functionality of oligo-modified IgG standard cell staining with these antibodies and QDs conjugated to secondary antibodies was done. As shown in Fig. 5 both AR (red staining with anti-mouse QD655) and MAOA (green staining with anti-rabbit

QD586) were detected, thus indicating that performed modification did not affect reactivity and specificity of antibodies.

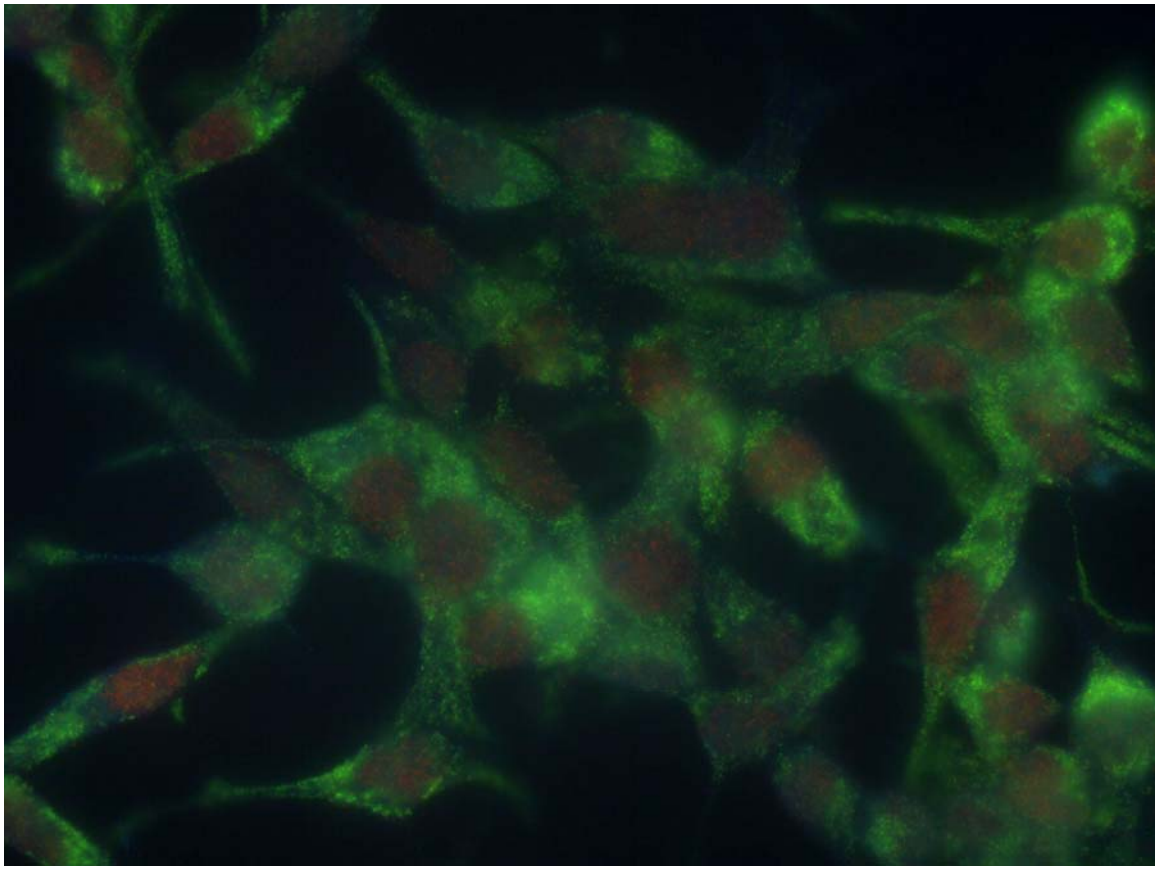


Fig. 5: Staining of LnCap cells with oligo-modified antibodies. Uniform nuclear staining of AR with QD655 (red) and patchy staining of MAOA with QD586 (green) indicate that modified antibodies retained their reactivity and specificity.

Prostate cancer molecular imaging with QD-oligo and IgG-oligo bioconjugates:

Prostate cancer LnCap cells were grown on round 12mm glass coverslips for 2-3 days to ensure complete cell recovery from trypsinization and normal expression of all biomarkers. Prior to staining cells were fixed with 7% formalin for 10 minutes, permeabilized with 2% DTAC/TBS for 20 minutes, and washed with TBS. IgG-oligo probes were applied to cells in 2%BSA/0.05%Tween-20/TBS solution and incubated for 1 hour at room temperature. Then excess probes were removed by washing with TBS. Reporter QD600-oligo probes were applied at QD concentration ~4nM in 2%BSA/Borate buffer and incubated for 1 hour at room temperature followed by washing with Borate buffer and dehydration in series of ethanol dilutions. Slides were coversliped using Cytoseal-60 medium and examined on fluorescence microscope within 1 hour (as coverslipping procedure caused QD quenching within several hours). As shown on figure 6, distinct staining pattern was obtained for both AR (nuclear) and MAOA (patchy) biomarkers as compared to standard staining

procedure with commercial QD-secondary Ab. Minimal non-specific staining was observed when no antibody or IgG with non-complimentary oligonucleotide were used. The expression of MAOA and AR was further confirmed with traditional proteomic techniques such as FISH and western blotting.

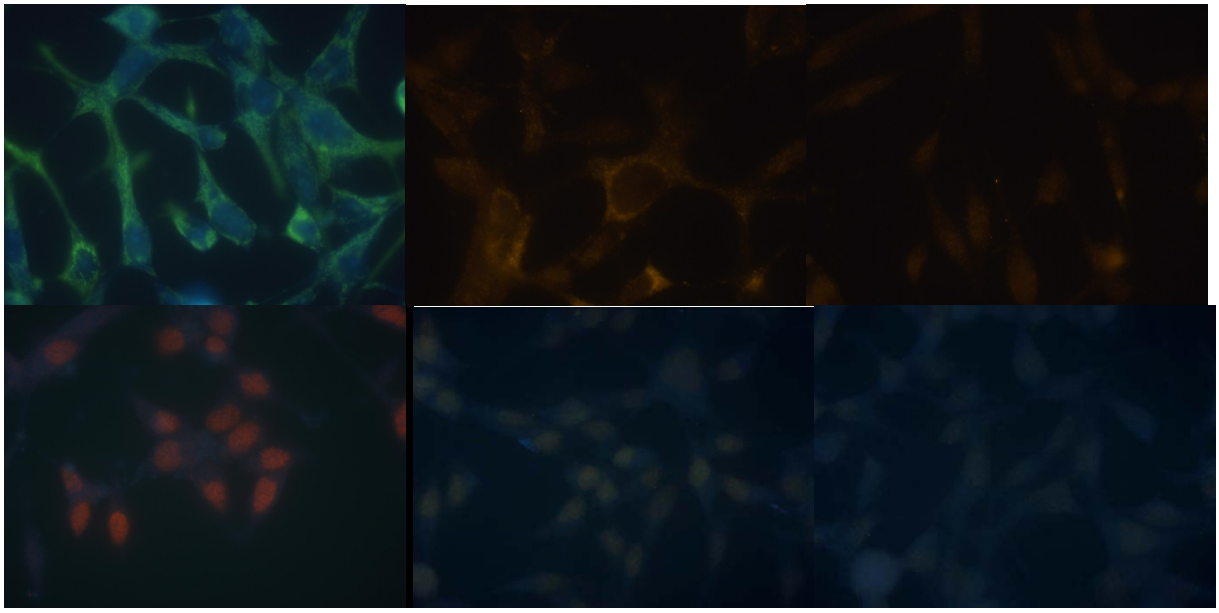


Fig. 6: Staining of formalin-fixed prostate cancer cells with QD-oligo and IgG-oligo bioconjugates. Patchy MAOA staining (top row) and uniform nuclear AR staining (bottom row) observed with standard staining procedure (left) was obtained only with complimentary QD-IgG pair (middle) and not in control (right).

Key Research Accomplishments

- Synthesis of multicolor quantum dot nanoparticles
- Identified two novel amphiphilic polymers for water-solublization of quantum dots and surface functionalization
- Ultracentrifugation is one effective method for purification of polymer-coated QDs from excess polymer. Approaches based on separation by size (such as gel column or concentrator) fail to remove excess polymer due to possible polymeric micelle formation comparable in size to QDs.
- Design and synthesis of oligonucleotides with similar T_m and minimum similarity to endogenous DNA in mammalian cells
- Primary amine groups on 5' end of oligonucleotides is probably hidden within PEG chains when exposed to aqueous environment due to hydrophobicity of C6 linker. Dissolution in DMSO or DMF aids in exposing primary amines for further modification.

- EDC-mediated conjugation of oligonucleotides to PMAL-coated QDs is effective in DMSO/DMF, producing functional bioconjugates with 3-4 oligos per QD and leading to slight increase in nanoparticle hydrodynamic size. Identical reaction in Bicarbonate buffer is ineffective.
- Conjugation of oligonucleotides to antibodies using SANH/SFB cross-linking effectively produces IgG-oligo bioconjugates with preserved antibody reactivity and specificity.
- Prostate tumor cells can be specifically stained using the proposal FINISH technology.

Reportable Outcomes

Publications

- P. Zrazhevskiy and X. Gao. Chapter "Molecular profiling of cancer cells and tissues using multicolor quantum dots" for "Nanomaterials: Inorganic and Bioinorganic Perspectives," edited by C. Lukehart and R. Scott, John Wiley & Sons, Ltd.
- P. Zrazhevskiy and X. Gao. Chapter "Bioconjugated quantum dots for tumor molecular imaging and profiling" for "Oxford Handbook of Nanoscience and Technology: Frontiers and Advances," edited by A. Narlikar and Y. Fu, Oxford University Press, UK.
- P. Zrazhevskiy and X. Gao. Quantum dots for cancer molecular imaging. Minerva Biotecnologica (Accepted).

Presentations:

- 1. "Engineering of quantum dot-antibody bioconjugates for molecular profiling", 236th American Chemical Society (ACS) National Meeting.
- 2. "Quantum dot-antibody bioconjugates for molecular profiling", 2008 Center for Nanotechnology (CNT) Student Symposium.

Conclusion

In conclusion, we have successfully developed a quantum dot based multiplexed molecular imaging technology for tumor cell profiling and met all the molestones proposed. We believe this technology could become the first clinical application of quantum dot nanotechnology and will find broad applications in molecular pathology.

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Appendices

N/A